pH-controlled recovery of placenta-derived mesenchymal stem cell sheets

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Abstract: Widely used in different biomedical applications, polyelectrolyte multilayers provide inter alia an attractive way for manufacturing of bio-functionalized, stimuli responsive surface coatings to control cellular behavior. In this study a novel polyelectrolyte-based platform for the engineering and controllable detachment of human mesenchymal stem cell (MSC) sheets is presented. Thin films obtained by layer-by-layer deposition of cationic poly(allylamine hydrochloride) (PAH) and anionic poly(styrene sulfonate) (PSS) polyelectrolytes on conductive indium tin oxide (ITO) electrodes allowed for the fast formation of viable sheets from human placenta-derived mesenchymal stem cells (PD-MSCs). Resulting stem cell sheets retained their phenotypical profile and mesodermal differentiation potency. Both electrochemically-induced local pH lowering and global decrease of the environmental pH allowed for a rapid detachment of intact stem cell sheets. The recovered stem cell sheets remained viable and maintained their capacity to differentiate toward the adipogenic and osteogenic lineages. This novel polyelectrolyte multilayer based platform represents a promising, novel approach for the engineering of human stem cell sheets desired for future clinical applications.

DOI: https://doi.org/10.1016/j.biomaterials.2011.02.058

Originally published at:
Guillaume-Gentil, O; Semenov, O V; Zisch, A H; Zimmermann, R; Vörös, J; Ehrbar, M (2011). pH-controlled recovery of placenta-derived mesenchymal stem cell sheets. Biomaterials, 32(19):4376-4384.
DOI: https://doi.org/10.1016/j.biomaterials.2011.02.058

1. Introduction

MSCs are multipotent cells present in a broad variety of adult human tissues **[1, 2]**. Emerging evidences show that human term placentas represent a rich, reliable, ethically uncontroversial, and relatively easily accessible source of maternal MSCs **[3]**. The therapeutic benefits of MSCs administered *in vivo* stem not only from their multiple differentiation capacities, but also from their migratory behavior, paracrine effects and unique immune-regulatory features **[4-7]**. A prerequisite for the repair of a damaged tissue with MSCs is that the stem cells can reach and engraft the target tissue **[1]**. Upon systemic injection, MSCs have the remarkable potential to spread in many tissues and home preferentially to sites of inflammation **[1]**. In contrast, the local injection of MSCs allows for the selective targeting of high cell numbers to the site of need and can be successfully applied even in cases where the blood flow is compromised.

In traditional tissue engineering approaches, MSCs are co-administered in combination with biomaterial scaffolds which control their localization. Cell sheet engineering provides an alternative approach for scaffold-free local cell delivery **[8, 9]**. In this method, confluent cell monolayers are released non-invasively from their substrate preserving the essential cell surface proteins and the extracellular matrix (ECM) assembled by the cells. The ECM under the recovered cell sheet not only supports the fragile free-standing cell monolayer, but also ensures the re-attachment of harvested cell sheets onto surfaces or other cells **[10]**. Therefore, the recovered cell sheets can be either directly transplanted to the host tissue or first assembled into a thicker 3D-tissues **[11, 12]**. By this approach the use of deleterious proteolytic enzymes can be circumvented, and the engraftment of biomaterials-free high density cell constructs without suture is achieved **[13]**.

The transplantation of MSC sheets on a scarred myocardium was shown to reverse wall thinning and improve cardiac function in rats with myocardial infarction **[14]**. Compared to the injection of dissociated cells, MSC sheets administered by intramuscular injection showed a better engraftment in the skeletal muscle **[15]** and the therapeutic efficacy was improved in treated myocardial infarction sites of rats **[16]**. Cell sheet technology represents thus a promising approach for cell-based therapy, minimizing adverse manipulations before transplantation and maintaining a favorable ECM environment to support the integration of transplanted cells into the host tissue.

Cell sheet engineering has originally been developed using cell culture dishes grafted with temperature responsive polymers that provide cell adhesive properties at 37°C and become non-fouling upon temperature shift below 32°C **[8]**. Later, alternative approaches for the release of cell sheets have been proposed, based on mechanic, electrochemical or magnetic triggers **[17-21]**. Among those, the electrochemical approach is particularly attractive due to high spatial and temporal precision, rapidity and reversibility, and the possibility for automation and remote control. Previous platforms designed for cell sheet release under electrochemical control are based on the controlled dissolution of sacrificial substrates made of self-assembled monolayers (SAMs) **[19, 22]** or polyelectrolyte multilayers (PEMs) **[23]**. These platforms offer versatile, biocompatible substrates, simple and inexpensive processing, precise spatially pattern control of cell organization, and are applicable to any surface geometry. PEMs in particular represent highly promising materials for the bio-interface, providing a precise control over their chemical, physical, and mechanical properties, and the possibility to incorporate bioactive moieties such as drugs, nucleic acids, peptides, proteins or growth factors **[24]**. PEM coatings composed of poly(allylamine hydrochloride) (PAH) and poly(4-styrene sulfonate) (PSS), two synthetic polyelectrolytes, recently emerged as excellent cell substrates. *In vitro*, PAH/PSS substrates showed good cell adhesive and cell proliferation properties for a variety of cell types including endothelial cells **[25, 26]**, fibroblasts **[27, 28]**, osteoblastic cells **[29]**, and hepatocytes **[30]**. They promoted adhesion and differentiation of endothelial progenitor cells derived from rabbit peripheral blood **[31]** or human umbilical cord-blood **[32]**, and improved cell growth and cell viability of bone marrow-derived human MSCs compared to fibronectin-modified surfaces **[33]**. Since all these studies revealed promising properties in cell culture, PAH/PSS coatings were recently evaluated *in vivo*. In a rabbit model, human umbilical arteries treated with PAH/PSS multilayers showed high potency for endothelial cell engraftment and no signs of inflammation during three months of implantation, holding great promises for small vessel replacement **[34]**.

When subjected to an electrochemical trigger, PAH/PSS were shown to be relatively stable, with thin films dissolving only slowly after a prolonged electrochemical stimulation. On the other hand the pH lowering, which results from electrochemically-induced water electrolysis at the electrode surface, was shown to extend a few hundreds of nanometer above the thin PAH/PSS films **[18]**.The cells cultured on PAH/PSS substrates most probably interact with a layer of proteins adsorbed on the polyelectrolyte films from the

growth medium or produced by the cells. The protein-substrate, protein-protein, and individual protein intra-molecular interactions result from the sum of multiple interactions of different nature (ionic, hydrophobic, hydrogen bonding, van der Waals, etc.). Subtle changes in the proteins microenvironment, such as a decrease in pH, are expected to induce significant reduction in the stability of adsorbed protein layer **[35]**. We therefore speculated that a local, electrochemically-induced pH lowering at the bio-interface could instigate cell sheet detachment, through perturbation of the protein ad-layer on which cells adhere.

The present work describes an approach for cell sheet engineering, where either local or global pH lowering allowed for controlled detachment of PD-MSC sheets. The stability of PAH/PSS substrates in the presence of low pH, and their suitability for the culture of PD-MSCs were examined *in vitro*. The compatibility of the harvesting process with regard to the preservation of stem cell viability, phenotype and differentiation potential was also evaluated.

2. Materials and methods

2.1 Polyelectrolytes and solutions

HEPES buffer was prepared with 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; Fluka, Buchs, Switzerland) supplemented with 150 mM sodium chloride (Fluka, Buchs, Switzerland) in ultra-pure water filtered through MilliQ Gradient A10 filters (Millipore AG, Switzerland), with a pH adjusted to 7.4 using 6 M NaOH (Fluka, Buchs, Switzerland). Cationic poly-(allylamine hydrochloride) (PAH; MW = 56000 Da) and anionic poly(sodium 4-styrenesulfonate) (PSS; MW = 70000 Da) were purchased from Sigma GmbH, Switzerland, and used in solution at 0.1 mg/ml in HEPES buffer. All solutions were filtered through 0.22 μm pore size filters before use.

2.2 Preparation of PAH/PSS coated ITO electrode substrates

Glass substrates coated with a 50 nm thick ITO layer (Microvacuum, Hungary) were cleaned by 10 minutes ultrasonication in isopropanol, 10 minutes ultrasonication in Millipore water, N_2-blow drying, and 2 minutes O_2 plasma treatment (PDC-32G, Harrick, USA) shortly prior to polyelectrolyte deposition. Sequential layering of the polyelectrolytes and rinsing were done using a custom made spraying system based on Lego Mindstorms NXT robotics kit (LEGO Group, Denmark), which design was described in our previous publication **[18]**. All deposited PEM layers were sprayed at a constant pressure of 1 bar. A first layer was adsorbed by 5 sec spraying of PAH solution. After 15 sec the remaining PAH solution was rinsed by spraying of HEPES buffer for 5 sec. Then a second layer was adsorbed by 5 sec spraying of PSS solution, 15 sec incubation, and subsequent rinsing for 5 sec. Adsorption of PAH and PSS was performed alternately until nine layer pairs were built up. The PAH/PSS coated chips were kept in HEPES buffer until further use.

2.3 Atomic force microscopy

A Nanowizard I BioAFM (JPK Instruments, Germany) and Mikromasch CSC38/noAl cantilevers were used. The PAH/PSS coated chips (24 mm × 24 mm × 0.5 mm) were mounted in a custom made electrochemical liquid-cell provided with a three electrode configuration system: the ITO surface of the substrate contacted with a metallic copper spring served as working electrode, a silver wire was used as

Ag/AgCl reference electrode, and a platinum wire as counter electrode. The samples were scanned both in contact and intermittent-contact fluid modes. At first, polyelectrolyte-coated samples were scratched with a razor blade in order to localize the underlying ITO surface. For assessing the stability of PAH/PSS coatings under electrochemical stimuli, imaging and electrochemical step were alternated, the samples were scanned at the same defined local areas at each imaging step. The electrochemical AFM experiments were performed in cell growth medium supplemented with 3 mM HEPES. The electrochemical stimuli (30 or 100 μA/cm²) were applied using an AMEL potentiostat/galvanostat (model 2053, AMEL electrochemistry, Italy). No rinsing of the samples was performed. To assess the coating stability under changes of the pH of the bulk solution, the samples were mounted in the same electrochemical liquid-cell but no electrochemical trigger was applied. After imaging the sample at several defined locations in cell growth medium at pH 7.4, the medium was exchanged with cell growth medium of pH 4 and the same local areas were imaged at different time points for 2 hours following the pH lowering. The obtained ecAFM height mode images were further processed using SPM image processing software (JPK Instruments, Germany).

2.4 Cell seeding isolation and characterization of PD-MSCs

All tissue samples were obtained after informed consent of the mothers. The experiments were approved by the ethical committee of the District of Zurich which operates under federal legislation. Isolations of PD-MSCs were performed as described previously **[3]**. Briefly, 25g biopsies were taken from the stromal compartment of the human placenta immediately after elective caesarean section, washed three times with PBS, minced, and digested with a mixture of 0.25% trypsin (Invitrogen, Switzerland) and 12.5 U/ml of collagenase I (Roche AG, Switzerland). The resulting cell suspension was filtered through 100 μm cell strainer (BD Biosciences, Switzerland), and centrifuged with 1200 rpm at RT for 10 min. The cell pellet was finally resuspend in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH, Germany) and the resulting cell suspension was plated out into tissue culture flasks (TPP, Switzerland).

If not stated otherwise, PD-MSCs were seeded at densities of 1 x 10^6 or 5 x 10^4 cells/cm^2 on the PAH/PSS coated ITO chips (10 mm × 20 mm × 0.5 mm). Prior to cell seeding, the polyelectrolyte-modified substrates were mounted in custom made Teflon mold. Till further manipulations the cells were cultured in

non-hematopoietic stem cell expansion medium (Miltenyi Biotec, Germany) under normal culture conditions at 37ºC and 5% CO_2. All cells and cell sheets were analyzed microscopically using a Zeiss Axiovert 200M microscope (Carl Zeiss, Switzerland) equipped with an AxioCam MRc digital camera (Carl Zeiss, Switzerland) and analyzed with AxioVision V. 4.5.0.0 software (Carl Zeiss, Switzerland).

2.5 Phenotypical characterization of PD-MSCs sheets grown on PAH/PSS by FACS

To phenotypically analyze PD-MSC grown in sheets, cell sheets were grown for 4 hours under standard cell culture conditions. After complete removal of the culture medium, cell sheets were washed once with PBS, and then dissociated by incubation with 0.25% Trypsin-EDTA solution (Invitrogen, Switzerland) at 37ºC and 5%CO_2 for 5 min. For FACS analysis 10^5 living cells per probe were suspended in FACS buffer (1% BSA in PBS). The cells were then incubated for 25 min at 4°C with fluorescently labeled primary antibodies HLA-ABC (BD Pharmingen, Switzerland), HLA-DR (BD Pharmingen, Switzerland), CD34 (Miltenyi Biotec GmbH, Germany), CD45 (Miltenyi Biotec GmbH, Germany), CD73 (BD Pharmingen, Switzerland), and unlabeled primary antibodies CD14 (BD Pharmingen, Switzerland), CD90 (BD Pharmingen, Switzerland), and CD105 (BD Pharmingen, Switzerland). Probes incubated with unlabeled primary antibodies were then additionally stained by incubation for 25 min at 4°C with a FITC labeled goat anti-human secondary antibody (BD Pharmingen, Switzerland). Unstained cells served as negative controls. After staining all probes were washed with FACS buffer, fixed with 4% buffered formalin, and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). A minimum of 10^4 gated events, were acquired for each sample.

2.6 Differentiation of PD-MSCs sheets manufactured on PAH/PSS substrates

The mesodermal plasticity of PD-MSC sheets was analyzed by their induction with commercially available differentiation media, according to the manufacturer's protocols [MACS guide]. Precisely, PD-MSC cultures of passage 2 taken from 3 different donors were seeded onto $[PAH/PSS]_9$ PEM coated ITO treated glass electrodes at density 5 x 10^4 cells per cm^2 and grown to confluence. Adipogenic, chondrogenic, and osteogenic differentiations were initiated with AdipoDiff, ChondroDiff, or OsteoDiff differentiation media (Miltenyi Biotec GmbH, Germany) respectively. Control cell sheets were grown under identical conditions but in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH,

Germany). For all conditions the medium was changed every 48 hours. During and after differentiation all samples were examined microscopically.

2.6.1. Adipogenic differentiation

Adipogenic differentiation of PD-MSC sheets was conducted for 21 days. At day 21 induction medium was removed and cell sheets were fixed in ice-cold 100% methanol for 5 min at -20ºC. After that cell sheets were washed once with deionized water and lipid droplets were visualized by staining with 0.5% solution of Oil Red O (Sigma-Aldrich GmbH, Switzerland) for 20 min at RT. Finally, stained cell sheets were washed three times with water and analyzed microscopically.

2.6.2. Chondrogenic differentiation

After 21 days of chondrogenic differentiation, the induction medium was removed and cell sheets were fixed in 4% paraformaldehyde for 20 min at RT. After that they were washed with PBS and collagen II expression was assessed by immunocytochemical staining with polyclonal goat anti-human collagen II antibodies (Santa Cruz, Ltd. USA) and secondary FITC-conjugated anti goat antibodies (DAKO Cytomation, Denmark). Cell nuclei were stained with 4'6- diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR).

2.6.3. Osteogenic differentiation

Osteogenic differentiation was conducted for 11 days, then the medium was removed and the cells were fixed in 70% ethanol for 40 minutes at RT. After washing with deionized water, the alkaline phosphatase expression of cells was visualized by cytochemical staining with FAST BCIP/NBT (5-Bromo-4cloro-3-indolyl phosphate/Nitro Blue Tetrazolium) (Sigma-Aldrich GmbH, Switzerland).

2.7 Electrochemically-induced harvesting of PD-MSCs sheets

For electrochemically-induced cell sheet detachment, PD-MSCs were cultured for 4 hours on PAH/PSS coated chips, which were then mounted in the electrochemical flow cell provided with a similar three-electrode configuration system as described before (Section 2.3). Non-hematopoietic stem cell expansion media (Miltenyi Biotec GmbH, Germany) was supplemented with 3 mM HEPES. A current density of 30

μA/cm² was applied using an AMEL potentiostat/galvanostat (model 2053, AMEL electrochemistry, Italy) while the samples were observed by phase contrast microscopy (DM IL, Leica Microsystems Ltd, Switzerland).

2.8 pH-mediated stem cell sheets release

Cells were plated out at density of 1 x 10^6 cells/cm^2 on $[PAH\text{-}PSS]_9$ PEM coated ITO treated glass electrodes and cultured for 4 hours. After that, the culture medium was removed and the cell sheets were washed with PBS. For pH-mediated cell sheet peeling, media (non-hematopoietic stem cell expansion media, Miltenyi Biotec GmbH, Germany) with different pH values (4, 5, 6, and 7) were prepared by addition of 1M NaOH or 6M HCl, respectively and controlled using Seven Easy pH-meter (Mettler Toledo GmBH, Switzerland) 1 hour after adjustment. The peeling of the MSC sheets was initiated by addition of media with different pH and monitored by time-lapse microscopy using Olympus IX81 (Olympus, USA) equipped with a digital camera F-View (Olympus, USA).

2.9 Live-dead staining of harvested stem cell sheets

Harvested PD-MSC sheets were analyzed for viability using live-dead staining procedure. For this, cell sheets were rinsed three times with PBS and then incubated with a mixture of 1 μM calcein acetate and 2 μg/ml ethidium homodimer for 30 min. After rinsing three times with PBS, images were acquired using Zeiss Axiovert 200M (Carl Zeiss, Switzerland) equipped with a digital camera AxioCam MRc (Carl Zeiss, Switzerland), and analyzed with AxioVision V. 4.5.0.0 software (Carl Zeiss, Switzerland).

2.10 Mesodermal differentiation of harvested stem cell sheets

Peeled PD-MSC sheets were transferred to 35 mm diameter Petri dishes (TPP, Switzerland) and allowed to adhere in non-hematopoietic stem cell expansion medium (Miltenyi Biotec GmbH, Germany) at 37ºC and 5% CO_2 for 24 hours. After that the culture medium was replaced with AdipoDiff, ChondroDiff, and OsteoDiff induction media (Miltenyi Biotec GmbH, Germany), respectively. Appropriate differentiation and staining procedures were performed according the manufacture protocols as described before (Section 2.6).

3. Results

3.1 Characterization and Stability of PAH/PSS thin films

Poly(allylamine hydrochloride) and poly(styrene sulfonate) PAH/PSS multilayered thin films were assembled layer-by-layer on ITO electrodes to generate substrates for the growth and subsequent harvesting of PD-MSC sheets. The deposition of nine bilayers of PAH/PSS on the electrodes yielded continuous thin films with a thickness of about 20 nm (Fig. 1 A and F). The stability of the PAH/PSS substrates in respect to an external electrochemical trigger was monitored by ecAFM. Under an applied current density of 30 μA/cm², the PAH/PSS films remained stable for at least 30 minutes, without any changes in thickness or surface roughness (data not shown). Applying a higher current density (100 μA/cm²), no changes in thickness or surface roughness were detected by ecAFM measurements within 30 minutes, while a small decrease in thickness indicating the dissolution of the coating was detected after 60 minutes (Fig. 1 A-E). The stability of PAH/PSS films was further assessed by lowering of the bulk pH. The coating, prepared under physiological conditions at pH 7.4, was immersed in a buffer at pH 4.0 for 2 hours. AFM images showed neither change in the coating thickness nor surface roughness (Fig. 1 F-H).

3.2 Formation of PD-MSCs confluent monolayers on PAH/PSS substrates

3.2.1 Growth and phenotypical characterization

To test the ability of PAH/PSS modified electrodes to allow for adhesion and formation of confluent PD-MSCs layers, cells were cultured at different densities on these substrates. The cells attached easily and exhibited fast proliferation on the polyelectrolyte substrates, indicating that normal cell anchorage was established. When seeded at high density ($5x10^5$ cells/cm^2), PD-MSCs formed confluent monolayers within 4 hours (Fig. 1A). The viability and phenotypical stability of the PD-MSC sheets grown on the PAH/PSS substrates or standard culture conditions on the TCPS plates were compared by live-dead staining and FACS analysis, respectively. Live-dead staining using calcein and ethidium homodimer showed that PD-MSCs in the confluent monolayers remained viable to a similar extent (95%) as under standard culture conditions (95%) (Supplementary Fig. 1). Phenotypical characterization performed according to the minimal criteria for determining of human MSCs (international society for cell transplantation (ISCT)) also did no reveal significant changes in surface marker expression in response to

confluent PD-MSCs cultured on the PAH/PSS substrates **[36]**. Indeed, the cells in both conditions were positive for HLA-ABC, CD73, CD90, and CD105 but negative for HLA-DR, CD14, CD34, and CD45 cell surface markers (Supplementary Fig. 2).

3.2.2 Mesodermal differentiation of PD-MSCs cultured on PAH/PSS

To further test whether PD-MSCs cultured as dense cell monolayers retained their potential to differentiate *in vitro* towards mesodermal lineages, cell sheets were subjected to adipogenic, chondrogenic, and osteogenic differentiation. Because mesodermal differentiation of MSCs typically takes up to three weeks, a stable adhesion of the cultured monolayers within this period was desired. PD-MSC monolayers remained stably attached to the PAH/PSS coated ITO surfaces for the whole period of culture in control as well as in osteogenic, and chondrogenic differentiation conditions. In contrast, the PD-MSC monolayers upon adipogenic differentiation (21 days) started to detach from the PEM substrates and rolled up (Fig. 5 B). As shown by specific biochemical and immunocytochemical staining, confluent cultures of PD-MSCs could be successfully differentiated towards mesodermal lineages (Fig. 5). The deposition of lipid droplets, expression of alkaline phosphatase, or expression of collagen type II were detected in cell sheets stimulated by adipogenic, osteogenic, or chondrogenic differentiation media respectively (Fig. 5 B, D and F). However, no sign of differentiation was observed for control, non-induced, PD-MSC monolayers (Fig. 5 A, C and E).

3.3 Electrochemical harvesting of PD-MSCs sheets

PD-MSC monolayers grown on PAH/PSS coated electrodes were subjected to an electrochemical trigger to induce the detachment of cell sheets. Although, PAH/PSS thin films did not show signs of disintegration at a current density of 30 μA/cm², using ecAFM analysis, under these electrochemical conditions the PD-MSC sheets completely detached from the substrate within 10 to 20 minutes (Fig. 2). Remarkably, the PD-MSCs sheets were released from the substrate as intact cell-sheets, indicating that the cells remained attached to their own ECM and maintained the established cell-cell connections. Furthermore, upon transfer to a new substrate a fast adhesion of the harvested cell sheets was observed, supporting the notion that the extracellular matrix proteins as well as cellular functions are not impaired during the electrochemical release from the substrate surface.

3.4 Release of PD-MSCs sheets by bulk pH decrease

One of the mechanisms driving the release of confluent cell sheets from PAH/PSS coated ITO electrodes during the application of an electrochemical potential could be a local drop of pH [18, 23, 37] .In order to study the influence of acidic conditions on the adhesion of the confluent PD-MSCs, cell-sheets were subjected to an environmental pH change. For this, PD-MSC sheets grown at physiological pH of 7.4 on PAH/PSS coated ITO electrodes were exposed to cell culture media buffered at pH values ranging from 7.4 to 4.0. Our time lapse evaluations demonstrated that cell attachment remained unchanged when the media was buffered above pH 5.0. However, when the pH value was reduced to 4.0, cells readily detached within 2-3 minutes as continuous and intact cell sheets (Fig. 3). These recovered cell sheets spontaneously attached when transferred onto new dishes.

3.5 Viability and mesodermal plasticity of harvested PD-MSCs sheets

The survival and functionality of PD-MSCs after release by electrochemistry and lowering of pH was tested immediately after the detachment by live-dead staining. As depicted in Fig. 4, live-dead staining performed within 2 hours after detachment demonstrated that the cell survival was not significantly compromised. Furthermore, our results confirmed that transferred cell sheets retained their potential for adhesion and growth on novel TCPS substrates (data not shown).

Finally the differentiation of the peeled PD-MSC sheets towards adipogenic and osteogenic lineages could be successfully shown by Oil Red O and Alizarin Red S staining, respectively (Fig. 6). The efficiency of this differentiation was comparable with the results obtained after mesodermal differentiation of confluent cell layers grown on PAH/PSS substrates deposited on ITO coated glass electrodes described above.

4. Discussion

In the present study, a platform for the growth and subsequent non-enzymatic harvesting of MSC sheets was explored. Robust PAH/PSS polyelectrolyte multilayer thin films (PEMs) were used as substrates for PD-MSCs culture and mesodermal differentiation. Furthermore, viable PD-MSC sheets were effectively released from these polyelectrolyte substrates through a local, electrochemically-induced pH lowering or a global pH decrease of the bulk solution. PAH/PSS coatings deposited on conductive ITO substrates

promoted the formation of confluent PD-MSC monolayers while preserving the viability and differentiation potency of the adult stem cells. The viable stem cell sheets harvested upon electrochemical trigger or reduction of the bulk pH were able to adhere onto new TCPS surfaces, and could be successfully differentiated towards adipogenic and osteogenic lineages.

In a previous study, native multilayers assembled from combinations of poly(L-lysine) and hyaluronic acid (PLL/HA), weak polyelectrolytes which form relatively soft and highly hydrated coatings, were shown to be poorly adhesive for PD-MSCs even after functionalization with fibronectin. Only highly cross-linked, and thus stiffer (PLL/HA) multilayers proved to be suitable substrates for MSC adhesion and proliferation **[3]**. In contrast PAH/PSS assemblies described in this study represent a dense, stratified structure resulting in much stiffer films, that need no cross-linking to become good substrates for adhesion and proliferation of a various cell types, including stem cells, **[25-34, 38, 39]**. In this work, PAH/PSS films served as substrates for formation of confluent PD-MSCs monolayers without affecting their viability, phenotypical stability and differentiation capacity, further substantiating the promising properties of PAH/PSS coatings for biomedical applications.

When subjected to a current density of 30 μA/cm^2, PD-MSC sheets grown on PAH/PSS films completely detached within 10 to 20 minutes. However, ecAFM experiments showed that the same electrochemical stimulus applied for 30 minutes was not sufficient to induce an apparent coating dissolution. This is in good agreement with a previous report which showed only a detectable slow decrease in coating thickness after 120 minutes at similar electrochemical conditions **[18]**. As the dissolution of polyelectrolyte assemblies has been previously shown to be dependent on the intensity of the electrochemical stimulus applied, the stability of PAH/PSS thin films was assessed under a current density of 100 μA/cm^2. Also using this increased electrochemical stimulus, the integrity of the thin films was maintained for at least 30 minutes. The electrochemically-induced degradation of polyelectrolyte multilayers is thought to rely on the neutralization of the electrostatic interactions within the assemblies, a continuous production of protons at the electrode surface derived from water electrolysis.

$2H_2O \rightarrow O_2 + 4\ H^+ + 4e^-$ (E^0 = 1.23 vs. Normal Hydrogen Electrode (NHE))

The destabilization of PAH/PSS assemblies, requiring a very high concentration of protons due to the low pKa value of PSS sulfonate groups (pKa~1), results in a delayed, slow dissolution. These results indicate that the cell sheet detachment observed in this study might not rely on the dissolution of the cell's underlying substrate, in contrast to the electrochemical approaches using other substrates for cell sheet engineering **[19]**.However, the presence of a cell monolayer on the PAH/PSS-coated electrode most probably results in an accumulation of the electrochemically-produced protons between the electrode surface and the cells. Therefore, the electrochemically-induced pH lowering is probably greater in the presence of cells compared to a bare PAH/PSS-coated electrode. In their modeling of the electrochemically-induced pH lowering, Gabi et al. estimated that a pH of about 4 is reached close to a bare electrode surface with the application of similar (30 $\mu A/cm^2$) electrochemical triggers, while the pH between a cell and the electrode under the same conditions would reach a value around 3 **[37]**. With a pKa 1 for the sulfonate groups of PSS 100 % and 99 % of the sulfonate groups are ionized at pH 4 and 3 respectively (Henderson–Hasselbalch equation), indicating that a significantly lower pH is required to efficiently destabilize PAH/PSS assemblies. Yet, the precise pH value obtained in the present experimental conditions remains unknown, as the influence of the adsorbed polyelectrolytes and proteins, of the buffer molecules, and the permeability of the cell monolayer are unascertained.

Independent of the degradation of the PAH/PSS thin films, it has been previously evidenced that the drop in pH induced by the electrochemical reactions can extend to apposed layers. Indeed, more sensitive, weak (PLL/HA) assemblies, deposited on the top of a PAH/PSS thin film were shown to dissolve readily upon electrochemical trigger **[18]**. It can thus be assumed that in this work the pH at the film/cells interface decreased upon electrochemical stimulation. Since the interface between the cells and the substrate consists of a rich and complex protein layer, a change in pH may affect and destabilize protein-protein and protein-substrate interactions, or the individual protein conformations **[40-42]**. Such perturbations in the ECM protein layer on which cells adhere for example via specific ligand integrin interactions may induce the detachment of the cells. To verify this hypothesis, cell adhesion in response to decreased in bulk pHs was evaluated. An environmental pH of 4.0 was found to induce a rapid detachment of the cell sheets, while pHs between 5.0 and 7.4 did not affect cell attachment on PAH/PSS substrates. The integrity of PAH/PSS films immersed in a solution at pH 4.0 was confirmed by AFM measurements, excluding a

substrate dissolution as a cause of cell detachment. These results confirmed that cell sheets can be harvested by a local (electrochemically-induced) or global lowering of the pH. The electrochemically-induced pH lowering led to a slower cell sheet detachment compared to a direct change of the cover medium pH, a delay most probably due to the time necessary for the production of sufficient amount of protons to reach a pH of ~4 above the polyelectrolyte substrate. Instigating the pH decrease by electrochemistry presents however several benefits compared to an exchange of the bulk solution. Indeed, the electrochemical trigger is fast, precise and reversible, it allows for a local pH change confined at the biointerface, and it offers further possibilities for automation and for micro-patterning.

It is well known that micro-environmental pH changes can significantly affect final the cell population density, reversal of contact inhibition of growth, or efficiency of cell fusion *in vitro* **[41]**. For this reason, for the maintenance and stable growth of most cell types the pH of cell cultures must be kept within relatively narrow limits. As previously reported by Taylor et al., the elevation of the environmental pH can lead to acceleration of cell movement and cause cytoplasm contraction, while the lowering of pH can retard and stop cell activity, causing apparent gelation of the protoplasm **[42]**. Cecarrini and Eagle also demonstrated that the growth rate as well as the maximum population doubling of normal, cancer, or virus-transformed cell types was markedly pH dependent. The optimal pH ranges varied from 6.9 for rabbit lens cells, to 7.8 for human fibroblasts **[40]**. The effect of electrochemically-induced pH lowering on the viability of cells grown directly on an electrode has been studied recently. It appeared that current densities up to 38 $\mu A/cm^2$ did not significantly affect cell viability, while 58 $\mu A/cm^2$ induced transient pore formation in the cell membrane, and an electrochemical trigger ≥ 76 $\mu A/cm^2$ induced apoptosis. Furthermore, environments below pH 2 also induced cell death **[37]**.

In this work, the effect of both local and global pH reduction on the viability and differentiation potential of the PD-MSCs was assessed. Live-dead staining as well as differentiation assays of the harvested MSC sheets demonstrated that a transient pH decrease for inducing the cell sheet detachment had no adverse effects on the cells. Beside their good properties for cell culture, the thin PAH/PSS films might also shield the cells from reactive products produced by the electrochemical reactions at the electrode surface. Indeed, beside reactive oxygen produced upon water electrolysis, the electrochemical stimuli may also induce the formation of amine reactive hypochloric acid **[37]**. These highly reactive moieties could have

cytotoxic effects on cells attached directly on the electrode. However, these products cannot diffuse through a PAH/PSS film but will react and be neutralized at the electrode/polyelectrolyte interface. Beside presenting excellent cell substrate properties and a protective effect against potential cytotoxic electrochemical products, the PAH/PSS thin film also offers additional prospects for tailoring cell culture prior to cell sheet harvesting, through the embedment of desired bioactive compounds in the polyelectrolyte multilayer assembly.

5. Conclusions

In this study, cell sheet engineering through a global lowering of the environmental pH and through an electrochemically-induced local pH decrease were both shown to be efficient and fast, while presenting no deleterious effect on the cells. In contrast to previous electrochemical approaches for cell sheet engineering, the here presented platform allowed for the non-enzymatic harvesting of cell sheets while preserving the integrity of the substrate. Based on AFM results which have shown any detectable disintegration of polyelectrolyte layers upon pH dependent and electrochemical cell sheet detachment, we expect that the recovered cell monolayers are free of polymer. Furthermore, the integrity of the polyelectrolyte substrate after cell sheet detachment allows envisaging for multiple usage of the same substrate. Based on cell culture compatible PAH/PSS substrates which are easily prepared, loadable with desired bioactive molecules, and suitable for any surface geometry and for a broad variety of cell types, the cell sheet engineering approach depicted in this article holds great promises for future application in regenerative medicine.

6. Acknowledgements

We thank Dr. Antoine Malek, Aida Kurmanavicius, and Esther Kleiner for the help with isolation and culture of placenta-derived PD-MSCs. This work has been supported by the Competence Centre for Materials Science and Technology (CCMX) of the ETH-Board, Switzerland and the European Community's AngioScaff Program contract 214402

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Figure 1. EcAFM based characterization of PAH/PSS thin films after electrochemical stimulation and bulk pH decrease. AFM Height mode images of a PAH/PSS film deposited on an ITO electrode (A), and subsequently subjected to 100 µA/cm² for 15 (B), 30 (C) and 60 (D) minutes. The profiles, corresponding to the white dashed line in each AFM images showed no significant changes at 15 and 30 minutes, while a slight decrease in thickness was observed after 60 minutes (E). AFM height mode images of a PAH/PSS film deposited on an ITO electrode in HEPES-2 buffer (pH 7.4) (F) and after 2 hours in a buffer at pH 4.0 (G). The profiles corresponding to the white dashed lines on AFM images (H) showed no changes under the change of bulk pH. The black areas on each image correspond to scratches allowing for thickness determination.

Figure 2. Peeling of PD-MSC sheets by electrochemical stimulation. PD-MSCs were grown to confluence on ITO substrates coated with a PAH/PSS thin film (A) and subsequently subjected to a current density of 30 µA/cm^2. After 5 minutes of electrochemical treatment (B), the confluent cells remained attached to the substrate, while detachment of the whole, intact cell sheet was observed after 15 minutes (C).

Figure 3. Detachment of PD-MSC sheets upon lowering of the bulk pH. Monolayers grown from PD-MSCs on ITO substrates coated with a PAH/PSS thin film remain stable at physiological pH (A) and neutral pH (B) while detach spontaneously within 2-3 minutes after decreasing of pH up to 4.0.

Figure 4. Viability of stem cell sheets after peeling. The viability of the cell sheets peeled by electrochemical stimuli (A) or decrease of bulk pH (B) as demonstrated by Live-Dead staining with calcein (green) specific for live cells and ethidium homodimer specific for dead cells.

Figure 5. Mesodermal differentiation of PD-MSC cell sheets cultured on PAH/PSS coated ITO substrates. Control stem cell sheets cultured in standard conditions without induction do not show any sign of differentiation (A,C, and E). Adipogenic differentiation as demonstrated by Oil Red O staining of lipid droplets (B), Osteogenic differentiation as demonstrated by Alizarin Red S-staining of calcification (D), Chondrogenic differentiation as demonstrated by immunocytochemical Collagen Type II and DAPI staining (F).

Figure 6. Mesodermal plasticity of stem cell sheets after peeling.

Osteogenic differentiation as demonstrated by Alizarin Red S-staining of calcification after electrochemically mediated peeling (A) and colorimetric staining of alkaline phosphatase after pH mediated peeling (C). Adipogenic differentiation as demonstrated by Oil Red O-staining of lipid droplets after electrochemically mediated peeling (B) and after pH mediated peeling (D).

Figure 1

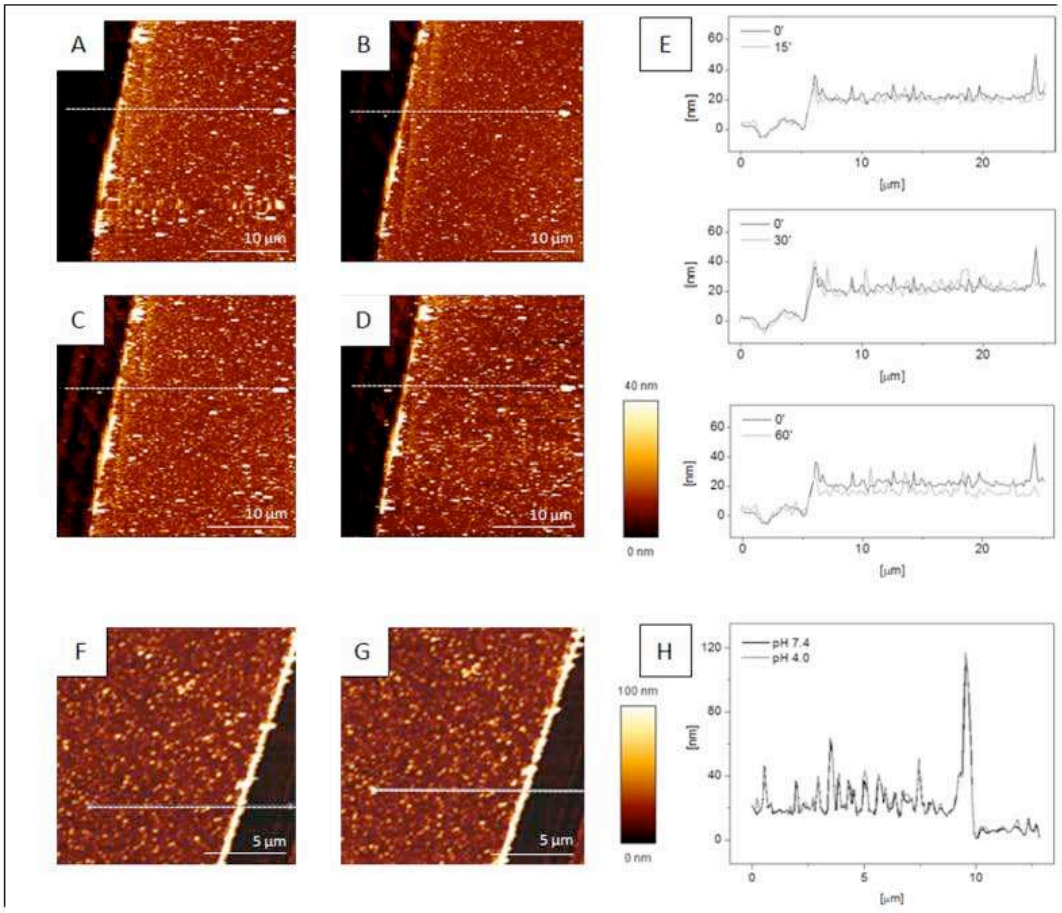

Figure 2

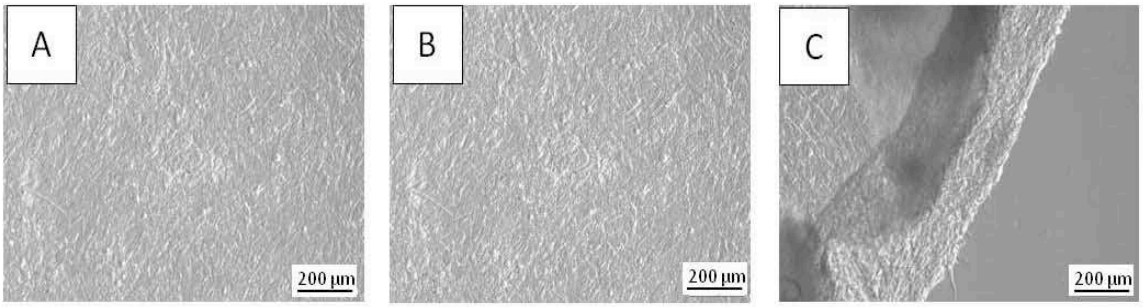

Figure 3

A

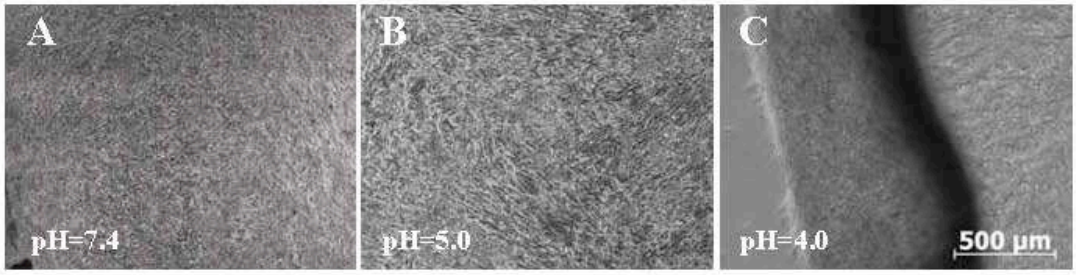

Figure 4

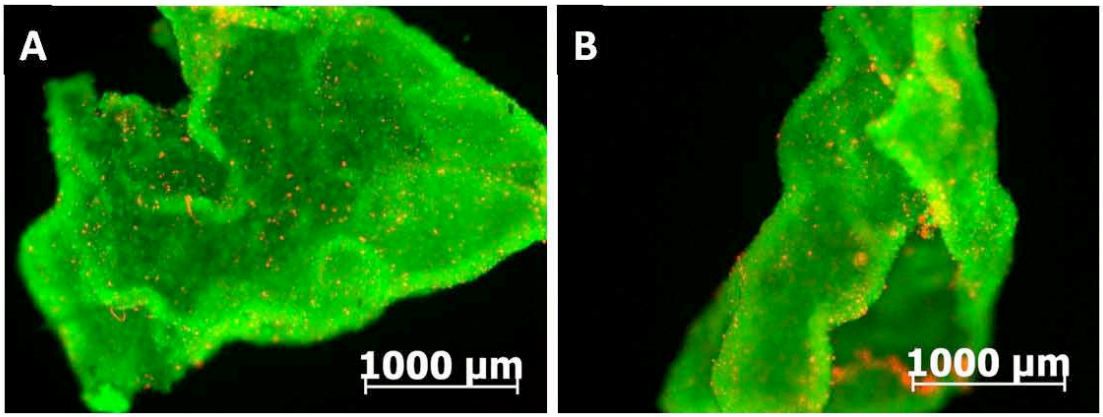

Figure 5

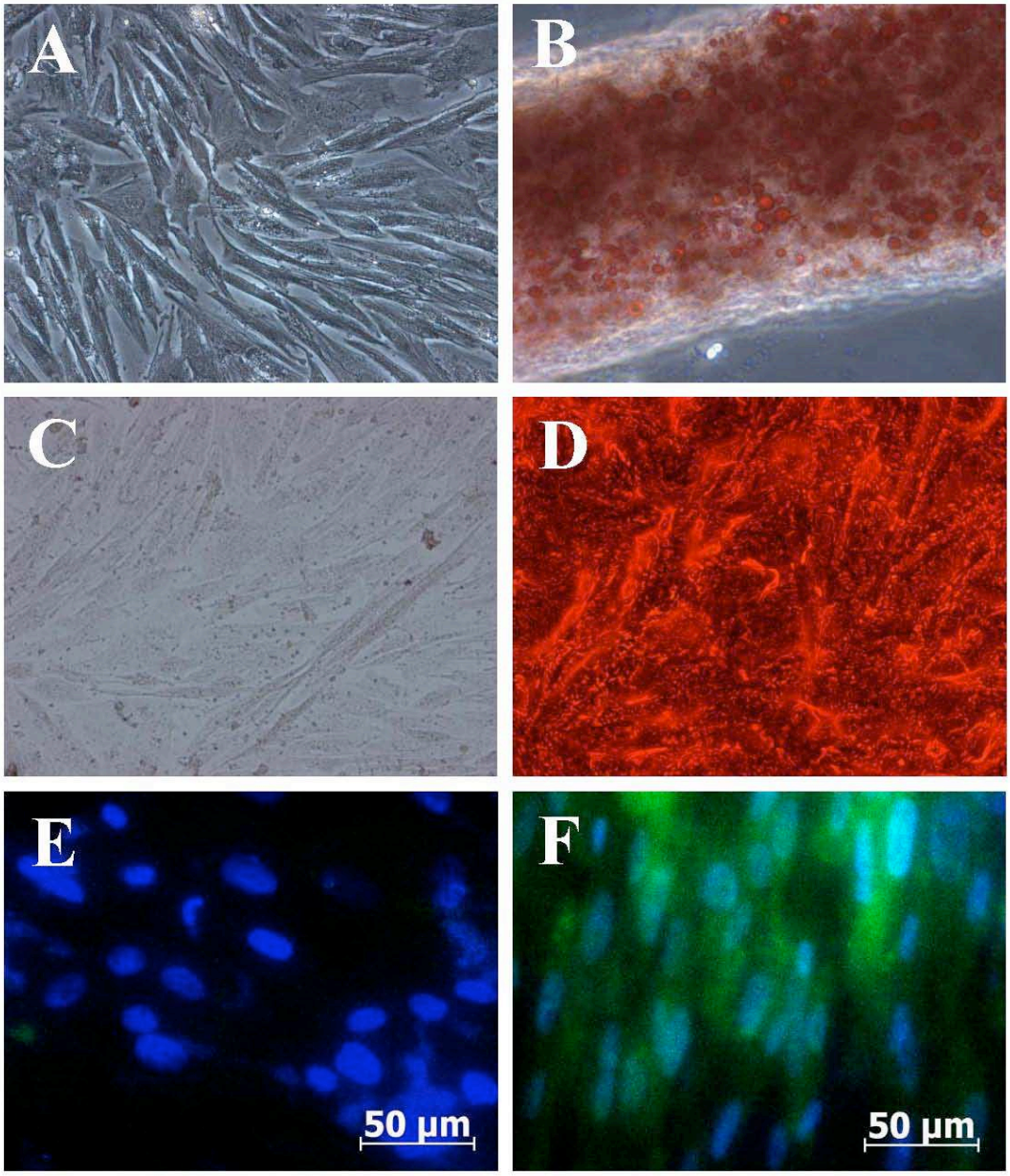

Figure 6